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Direct Fluorescent Decay Measurements Using High Speed Electronics

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Abstract

A high speed data acquisition architecture is implemented as part of a time-resolved fluorescence detection instrument to directly measure the time course of fluorescent decay. The architecture is implemented using a very fast dynode chain photomultiplier tube and associated gating circuitry, a broad spectrum light emitting diode excitation source, very wideband electronics for amplification and filtering, and a high speed digital oscilloscope. The fluorescence decay of tris (2,2′-bipyridyl) ruthenium (II) is measured and the lifetime measurement is compared with that using other reported methods. The system architecture is thereby validated for data acquisition of broadband signals including transient fluorescent recording.

Keywords: Data acquisition system; Ultra-wide band; Transient fluorescence recording; Fluorescent lifetime; Tris (2,2′-bipyridyl) ruthenium (II)

Introduction

Ultra-wideband data acquisition systems have recently become feasible with the advent of Giga-Sample per Second (GSPS) Analog to Digital Converters (ADC) and ultra-high speed analog electronics. There is a host of challenges associated with creating ultra-wide band systems. In order to give a high level view of these challenges, various basic electrical engineering terms, such as bandwidth, noise, and gain, will be discussed in reference with one another. The bandwidth of a system refers to the difference between the fastest changing and the slowest changing signals that the system is capable of detecting. The bandwidth is often referred to in terms of Hertz (e.g. Hz, kHz and MHz) and gives the range of usable frequencies that can be detected with minimal attenuation. [e.g.: The bandwidth can be defined for any attenuation. The most common definitions are for 0.1dB flatness where the pass-band has little to no attenuation and the -3dB bandwidth which corresponds to ½ the power being transmitted to the load.]

Tradeoffs exist in analog signal processing between gain, noise, and bandwidth. As the bandwidth increases so does the noise power bandwidth; as a result, for a given noise source density and gain, as the bandwidth increases the signal-to-noise ratio will decrease. Furthermore, increasing the gain will result in both a bandwidth reduction and an increase in output noise. Beyond those architectural limitations, parasitic impedance will limit both the achievable gain, and bandwidth. Unintentional parasitic impedance elements, negligible in most applications, such as the metal pads to which circuit elements are soldered or even metal traces used to connect various components can provide tremendous challenges in achieving the targeted specifications. The maximum achievable bandwidth of a single stage transimpedance amplifier using current technology with a transimpedance gain of about 30kΩ is between 10MHz and 50MHz [1]. Parasitic input and feedback capacitance in these amplifier circuits are the primary limiting factors. As transistor size continues to decrease, the achievable bandwidth number will continue to rise.

To achieve similar gain simultaneously with wider bandwidths, the initial transimpedance gain is reduced and is followed by additional amplification stages. This in turn reduces the achievable SNR (Signal-to-Noise Ratio). To achieve 1GHz bandwidths, the transimpedance and voltage gains are so low (of a multistage architecture) that it becomes impractical to amplify the signals more than 100 to 1000 times. However, overall gains on the order of 10,000 are necessary for many applications because GSPS ADCs cannot detect very small signals due to the limited number of bits available.

In addition, as the bandwidth increases, it is necessary to sample the signal faster. The power dissipation associated with GSPS rates for the latest technologies can be in excess of 4 W resulting in stringent thermal considerations [2]. Modern ADCs quickly become warm to the touch, requiring heavy external heat-sinks, and the associated power consumption makes it difficult to develop battery powered, ultra-wide band devices.

A final challenge is the data acquisition speed. Terabytes of data can literally be collected in only a few minutes. Intelligent data collection methods must be adopted in order to make the data tractable for signal processing and interpretation.

Ultra-wide band instrumentation for fluorescence spectroscopy

Ultra-wide bandwidth instrumentation is necessary in order to directly measure the time course of fluorescent decay signals. Fluorescence spectroscopy is a powerful tool for studying biological systems, minerals, and chemicals. Many substances fluoresce in a particular spectral range and have a specific decay rate making them identifiable via fluorescence spectroscopy. Additionally, the interactions between molecules frequently affect their fluorescence properties making fluorescence detection a valuable tool. Fluorescence detection methods are particularly suited to study biology. Intrinsic fluorophores in cells allow the detection and quantification of metabolites, DNA,

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RNA, and other biological molecules [3]. Extrinsic fluorophores can be bound to various non-fluorescent molecules as well, allowing the study of a wide variety of cellular processes [4].

Using time-resolved fluorescence to study viable biological samples has historically been challenging for two reasons. First, the samples are unstable and constantly changing. Second, exciting the sample repeatedly can cause photobleaching and other light-initiated reactions disturbing the natural dynamics of the sample.

We propose directly measuring fluorescent decay signatures via ultra-high speed electronics in order to minimize damaging the sample while simultaneously collecting data sufficiently quickly so as to capture the dynamics of the sample. We also propose using LED excitation sources with emission spectrums that are relatively broad compared to lasers in order to excite a larger number of the available fluorophores. Modern time-correlated single photon counting (TCSPC) [4] methods require hundreds of thousands of excitation pulses which can easily damage a biological sample. TCSPC also requires long periods of data collection during which time the sample can change. Frequency based methods require continuous excitation which can be even more damaging.

Our technique is a gating method because we are turning on a Photomultiplier Tube (PMT) directly after exciting a sample and recording the fluorescence generated by the pulse for the duration of the fluorescent decay (Figure 1). One of the main advantages of the gating technique is the increased dynamic range of the instrument. When a sample has a very low concentration, a high intensity excitation is required to produce a strong enough emission to be detected. PMTs can easily be saturated by the excitation light due to scattering, even if the excitation is set at a 90 degree angle from the detector. When a PMT is saturated, the PMT requires a certain amount of time to recover before it can again accurately detect light intensity. By gating the PMT on after the excitation pulse, no scattering is detected and the detection limit is significantly reduced.

There are several limiting factors associated with the instrument. First, fast electronics are currently limited to practical bandwidths of around 600MHz. The frequency spectrum of a single exponential decay curve is precisely the same shape as the frequency response of a first order low pass filter. If we approximate the system as being a first order low pass filter with a cutoff frequency of 600MHz, then the time constant describing the first order system (1/[2π*600MHz]) is shorter than the shortest detectable fluorescence lifetime. The shortest detectable lifetime must be longer than 1/(2π*600MHz) = 265ps. The fastest rise/fall times for LEDs due to driving circuitry limitations is approximately 500ps, which means that the LEDs can produce signals with bandwidths of approximately 0.35/(500ps) = 700MHz. (The equation used here is a rule of thumb frequently used in electrical engineering and is very precise for first order systems). If the electronics could support higher than 600 MHz bandwidths, they would still be limited by LED excitation allowing for a minimum detectable lifetime greater than 1/(2π*700MHz) = 227ps. The fastest PMTs on the market are microchannel plate (MCP) PMTs and typical rise times are around 150 picoseconds, making the maximum bandwidth around 0.35/(150ps) = 2.3GHz. The transit time spread of MCP PMTs is on the order of tens of picoseconds [4,5]. Unfortunately, to use these MCP PMTs for recording transient signals requires gains on the order of ten million which is currently impractical from a circuits design standpoint for bandwidths of 2.3 GHz.

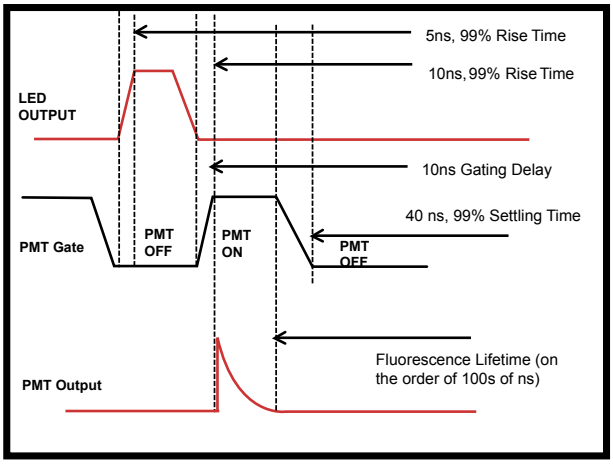

Figure 1: Timing diagram of a gated PMT detector. This diagram is only an example and does not reflect the exact timing of the instrument.

Instrument architecture

The instrument has six major components: an LED driver and LED, a PMT and associated gating circuitry, an amplifier and filter circuit, an analog to digital converter (ADC), a Field Programmable Gate Array (FPGA), and a Digital Signal Processor (DSP) board or computer (Figure 2). We have not optimized the optics, but this will be done for a field-portable instrument in the near future.

The LED driver provides adjustable power (light intensities) and pulse widths to excite a sample. The resulting fluorescence is collected by the PMT which amplifies the signal by a factor of 10^6 to 10^8. The PMT gating circuit is based on Molchanov's design allowing for nanosecond gating times [6]. Hamamatsu also provides examples of gating circuits [7].

Dynode chain PMTs have a maximum output current of about 0.1mA. Because the PMT signals are too small to read directly, a transimpedance amplification stage is required in order to convert the signal to a voltage and also to fill the range of the ADC. The amplification stage also filters the signal to minimize aliasing from frequencies that cannot be adequately sampled by the ADC.

High speed ADCs are all differential and generate data so fast that it has become necessary to catch and store the data with FPGAs before the data can be processed. We have taken advantage of that fact and used FPGAs to do 'on the fly' averaging before the data is stored. Lastly, the data is transferred to a DSP board or to a computer for final processing.

Preliminary instrument test results and data analysis

The prototype instrument has been initially low pass filtered to pass frequencies up to 10MHz, making fluorescent lifetimes longer than about 1/(2π*10 MHz) =15.9ns detectable. The output of the amplification stage was connected directly to a sampling oscilloscope. Figure 3 shows the output signals of the amplifier circuit, the LED

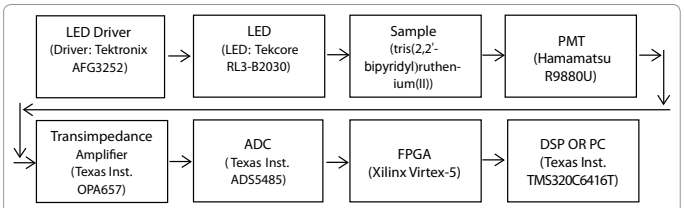

Figure 2: Diagram of the Instrument Architecture

driving signal voltage and the PMT gating voltage. The LED is the commercially available RL3-B2030 from Tekcore with a peak wavelength of 470nm and a typical brightness of 2000mcd. The blue curve is the instrument response when no fluorophore is present and the purple curve is the instrument response when a fluorophore, tris (2,2´-bipyridyl) ruthenium (II), has been excited by the LED. (Tris (2,2´-bipyridyl) dichlororuthenium (II) hexahydrate from Sigma-Aldrich was dissolved in water and used in the experiments).The concentration of tris (2,2´-bipyridyl) ruthenium (II) was measured to be 0.17mM. These signals have been collected 512 times and averaged to reduce the noise effects.

Subtracting the blue signal from the purple signal reveals the fluorescent decay data of tris (2,2'-bipyridyl) ruthenium (II). This data was fit to an exponential decay curve using the Trust-Reflective-Region Nonlinear Least Squares Algorithm as implemented by Matlab™ (Figure 4).

Discussion

The resulting exponential decay curve fit has a lifetime of 532ns. Some reported lifetimes in the literature for an aqueous solvent are 620ns [8] and 469ns [9]. The discrepancies in the lifetime measurements and our results may be due to the differences in oxygenation and/or temperature [which drastically affects the lifetime] or simply to the fact that the literature [8] reports this lifetime by using TCSPC when the technique was still new (1982) and relatively few counts were typically collected.

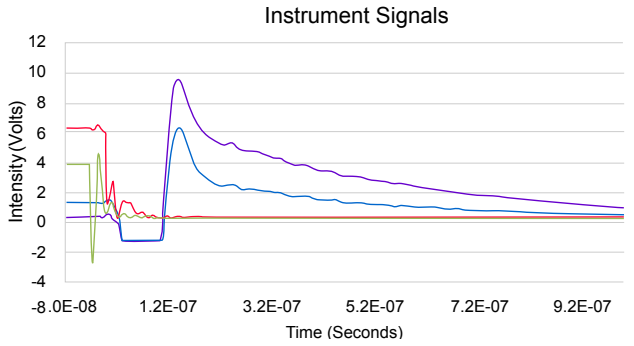

Figure 3: Fluorescence decay data of tris(2,2'-bipyridyl)ruthenium(II) obtained by gating a PMT When the green signal goes low, the LED turns off. Shortly thereafter, the PMT is gated on (the red signal goes low). The fluorescent decay is seen by subtracting the baseline (blue signal) when the fluorophores is not present from the signal when the fluorophores is present (purple signal). (Only a part of the time course is shown here.)

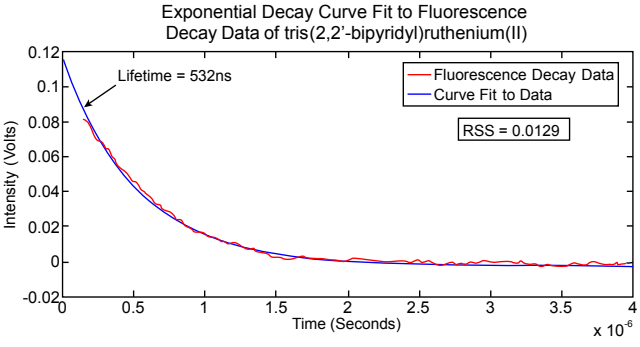

Figure 4: Fluorescence decay data of tris(2,2'-bipyridyl)ruthenium(II) fit to a single exponential decay curve, Matlab TM Trust-Reflective-Region Nonlinear Least Squares Algorithm is used to fit a curve to the data. The Residual Sum of Squares Error is equal to 0.0129.

The reference signal and the data were each collected in 512*(1/55kHz) = 9.3ms (repetition rate: 55kHz). The repetition rate could be as high as about 1/(5*532ns) = 376kHz and the noise could be reduced using careful circuit layout and shielding permitting less averaging. Thus, only tens to hundreds of microseconds would be necessary to obtain the decay time. For molecules with shorter lifetimes even less time will likely be required because the repetition rate can be increased.

Obtaining accurate, time-resolved fluorescent data lifetimes in the hundreds of nanoseconds range using the architecture described is possible. The architecture can be enhanced to potentially achieve fluorescent lifetime measurements on the order of a few nanoseconds by using a fast switching LED driver, ultra-wide bandwidth electronics, and ultra-high speed sampling. An enhanced architecture will likely yield instruments suitable for studying the dynamics of biological systems with time resolutions in the microsecond range. This method makes in-the-field applications such as micro-habitat and water supply monitoring possible.

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